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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT).

(51) International Patent Classification 5 :
C12N 15/82, C07H 21/04, A01H 5/00

A1

(11) International Publication Number: WO 94/25613

(43) International Publication Date: 10 November 1994 (10.11.94)

(21) International Application Number: PCT/US94/04557

(22) International Filing Date: 3 May 1994 (03.05.94)

(30) Priority Data:
08/054,362 3 May 1993 (03.05.93) US

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(81) Designated States: AU, BB, BG, BR, BY, CA, CN, CZ, FI, HU, JP, KP, KR, KZ, LK, LV, MG, MN, MW, NO, NZ, PL, PT, RO, RU, SD, SK, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: ISOLATED DNA ELEMENTS THAT DIRECT PISTIL-SPECIFIC AND ANTHER-SPECIFIC GENE EXPRESSION AND METHODS OF USING SAME

(57) Abstract

Isolated DNA elements that direct either pistil-specific or anther-specific expression of a polypeptide-encoding gene are disclosed. Plants comprising the DNA fragment operably linked to a gene are also disclosed. A further embodiment comprises a method of producing a female sterile plant, the method comprising growing a plant having integrated into its genome an isolated DNA element that directs pistil-specific expression of a nucleotide sequence that encodes or is transcribed into a moiety that causes female sterility in plants.

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ISOLATED DNA ELEMENTS THAT DIRECT PISTIL-SPECIFIC AND ANTHER-SPECIFIC GENE EXPRESSION AND METHODS OF USING SAME

The making of this invention was supported in part by funds from the United States Government. Therefor the United States Government might have certain rights to this invention.

FIELD OF THE INVENTION

The present invention relates to directing pistil- and anther-specific gene expression in transformed plants. In particular, the present inventors have established the minimal sequence elements of the S-locus glycoprotein (*SLG*) gene promoter necessary for this expression pattern.

BACKGROUND OF THE INVENTION

Self-incompatibility, a mechanism that prevents self-fertilization in flowering plants, is based on the ability of the pistil to recognize the presence of self-pollen and on the female tissue's capacity to restrict the growth or germination of self-related but not of genetically unrelated pollen. Although the mechanism of self-recognition is not yet known, a chromosomal location, the S-locus, has been shown from genetic crosses to encode the putative pistil and pollen elements that interact in this recognition process. When both the captured pollen grain and the receptive pistil possess and express different S-locus haplotypes, pollen growth proceeds; when the two S haplotypes are identical, pollen growth, as well as self-pollination, are prevented. The expression of

S-locus encoded elements in two tissues, the female sporophytic tissue as well as in the anther or pollen (or in both male tissues), is a hallmark of all models of self-incompatibility and distinguishes gene regulation in this system from many other systems in which tissue specificity is limited to a single location.

Self-incompatibility has been best studied in the Brassicaceae and Solanaceae, and genes associated with the S-locus have been identified in species of *Brassica*, *Nicotiana*, *Petunia*, and *Solanum*. In *Brassica*, two partially homologous genes have been shown to reside at the S-locus: one, *SLG*, encodes a secreted glycoprotein that is present in stigma papillar cells and in the tapetum and microspores of the anther, and the other, *SRK*, specifies a putative membrane-spanning receptor protein kinase that is present in *Brassica* pistil and anther. Expression of the S-locus products in the Brassicas is controlled sporophytically. A haploid pollen grain will possess a self-incompatibility phenotype that is determined by the two S-locus alleles carried by the parent plant. In contrast, the self-incompatibility phenotype of pollen derived from plants that express gametophytic control of self-incompatibility (e.g., in the Solanaceae) is dictated by the single S-locus haplotype carried by the pollen.

Evidence for this specificity of *SLG* promoter activity derives from genetic ablation studies in which a chimeric gene construct consisting of the *SLG* promoter fused to the diphtheria toxin subunit A (DTA) gene was introduced into tobacco (M.K. Thorsness, M.K. Kandasamy, M.E. Nasrallah and J.B. Nasrallah, Dev. Biol., Vol. 143, (1991) pages 173-184), *Brassica* (M.K. Kandasamy, M.K. Thorsness, S.J. Rundle, J.B. Nasrallah and M.E. Nasrallah,

Plant Cell, Vol. 5, (1993) (in press)), and Arabidopsis (M.K. Thorsness, M.K. Kandasamy, M.E. Nasrallah and J.B. Nasrallah, Plant Cell, Vol. 5, (1993) (in press)). Transformation of these plants with the SLG-DTA gene fusion resulted in the production at high frequency of transgenic plants that underwent normal differentiation and produced flowers in which only specific cells of the pistil and anther were ablated.

Surprisingly, when the *Brassica SLG* gene is introduced into *Nicotiana tabacum*, a self-compatible species belonging to the Solanaceae family, it is expressed in pistils in a manner similar to that noted for the S-linked RNase of *Nicotiana alata* and not according to the pattern shown for this gene in *Brassica*. In transgenic tobacco, the product of the introduced *Brassica SLG* gene accumulates in the secretory zone of the stigma, the transmitting tissue of the style, and to a lesser degree in the placental epidermis of the ovary. In *Brassica*, endogenous *SLG* molecules are detected mainly in the cell wall of stigma papillar cells. These expression patterns are consistent with the site and timing of self-pollen rejection in *Brassica* and *Nicotiana*.

Similar findings have been made with regard to S-locus expression in the anther. The analysis of the SLG-DTA fusion and of a reporter gene fusion consisting of the *SLG* promoter fused to the reporter β-glucuronidase *(GUS)* gene have identified the cell types of the pistil and anther in which the *SLG* promoter is active. In the pistils of transgenic *Brassica* and *Nicotiana*, the promoter is active in cells of the stigma and in the transmitting tissue of the style and ovary (T. Sato, M.K. Thorsness, M.K. Kandasamy, T. Nishio, M. Hirai, J.B. Nasrallah and M.E.

Nasrallah, Plant Cell, Vol. 3, (1991) pages 867-876; M.K. Thorsness, M.K. Kandasamy, M.E. Nasrallah and J.B. Nasrallah, Dev. Biol., Vol. 143, (1991) pages 173-184). In the anthers of transgenic *Brassica*, promoter activity is evident in the tapetum, a sporophytic tissue of the anther, and in microspores (T. Sato, M.K. Thorsness, M.K. Kandasamy, T. Nishio, M. Hirai, J.B. Nasrallah and M.E. Nasrallah, Plant Cell, Vol. 3, (1991) pages 867-876). In transgenic tobacco anthers on the other hand, the SLG-GUS fusion exhibits strict gametophytic expression: *GUS* activity is detected in pollen grains and not in the sporophytic tissues of the anther (M.K. Thorsness, M.K. Kandasamy, M.E. Nasrallah and J.B. Nasrallah, Dev. Biol., Vol. 143, (1991) pages 173-184). Moreover, approximately one half of the pollen grains of transformed plants that contain a single copy of the introduced gene show *GUS* activity. On the other hand, *Brassica* plants transformed with this construction display *GUS* activity in the tapetum, a sporophytic tissue of the anther, and in pollen microspores. Thus, although the S-locus-linked genes identified to date differ in plants possessing sporophytic and gametophytic forms of self-incompatibility, a common, conserved mechanism apparently exists in *Brassica* and *Nicotiana* for directing the expression of S-locus genes.

The DNA sequences required in *cis* for the expression of the *SLG* gene lie within 3.65 kb upstream of the gene's coding region. Promoter activity is detected in both pollen and pistil of transformed *Brassica* and *Nicotiana* plants and follows the temporal, spatial, and developmental-regulated expression pattern noted above.

SUMMARY OF THE INVENTION

Accordingly, one object of the present invention is to demonstrate that there are distinct *cis*-acting elements that direct pistil-specific and anther-specific activity and to define these elements.

Another object of the present invention is to define the uses of the elements to produce plants that express a specified gene in only the pistil or anther.

The present invention is drawn to isolated DNA elements which are capable of directing pistil-specific expression of a nucleotide sequence, when the sequence is operably linked to a minimal promoter. In this manner, minimal promoters can be utilized in combination with the DNA elements of the invention to direct pistil-specific expression of a nucleotide sequence of interest.

DNA elements are also provided which are capable of directing anther-specific expression.

The DNA elements of the invention can be utilized in combination with promoters, preferably minimal promoters, and nucleotide sequences, including sequences encoding polypeptides of interest and anti-sense RNA.

The present invention further provides methods of selectively expressing a gene in plant pistils or plant anthers, the methods comprising growing a plant having integrated into its genome the above-described isolated DNA elements, or functional equivalents of the isolated DNA elements, operably linked to a nucleotide sequence that is operably linked to a promoter.

In a preferred embodiment, the method produces a female sterile plant or a male sterile plant by operably linking the DNA element that directs

pistil-specific expression or anther-specific expression, respectively, to, e.g., a gene that encodes a moiety that is cytotoxic to plant cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts sequential 5′ deletions of the *SLG*$_{13}$ promoter fused to the β-glucuronidase *(GUS)* indicator gene. The 5′ endpoints of the promoter deletions are shown in relation to the *SLG*$_{13}$ promoter (top). The deletion endpoints are numbered from the translation initiation codon of the *SLG*$_{13}$ promoter.

Figure 2 depicts PCR-generated promoter modules derived from the *SLG*$_{13}$ promoter. The promoter modules were joined to the "TATA box" region of the CaMV 35S (-46/35S) promoter and to β-glucuronidase. The 5′ and 3′ endpoints of the promoter modules are shown in relation to the *SLG*$_{13}$ promoter (top).

Figure 3(A) (SEQ ID NOS:6-9) shows the nucleotide sequence and alignment of portions of the promoter regions of the *SLG*$_{13}$, *SLG*$_{2}$, *SLG*$_{8}$, and *SLR1* genes. The boxes outline five regions that show particular conservation among the four sequences. The sequences are numbered from the translation initiation codon of each gene. Figure 3(B) (SEQ ID NO:10) shows the nucleotide sequence of a portion of the promoter region of the *SLG*$_{13}$ genes 5′ adjacent to the portion shown in Figure 3(A).

Figure 4 is a diagrammatic representation of the arrangement of functional elements within the *SLG*$_{13}$ promoter.

DETAILED DESCRIPTION OF THE INVENTION

The present invention identifies minimal promoter elements required for the expression of a gene in the pistil or anther. Further, while the invention is described by reference to the S-locus glycoprotein *(SLG)* gene promoter and the S-locus related *(SLR1)* gene promoter of *Brassica*, it is understood that the invention is not limited thereto. Also, while transgenic tobacco plants were used as the experimental system, and while there are minor species-dependent differences in the activity of the *SLG* promoter, it is understood that tobacco is well suited for the functional dissection of this promoter because the dual activity of the promoter in pistil and pollen is maintained in this easily transformable plant.

To find pistil- and anther-specifying domains of the *SLG* promoter, several truncated versions of the promoter taken from the SLG_{13} allele were constructed. Sequential 5′ deletions of the promoter, or short promoter modules inserted upstream of a minimal promoter derived from the cauliflower mosaic virus (CaMV) 35S promoter (P.N. Benfey, L. Ren and N.H. Chua, EMBO J., Vol. 9, (1990) pages 1677-1684), were used to direct the expression of the *GUS* reporter gene in transgenic tobacco. The results established that distinct promoter elements direct *GUS* activity in pistil and pollen. A 196 bp region (-339 to -143) is sufficient to confer pistil-specificity to the marker gene. Two distinct, but functionally redundant, domains (-415 to -291 and -117 to -8) allow expression of the gene in the anther.

As used herein, the term "isolated" with respect to DNA elements, means DNA elements that are not in their natural environment. That is, the

DNA elements are not in a full length DNA strand found in nature. The element might have been isolated from the full length DNA strand via experimental techniques, such as use of restriction endonuclease enzymes and cloning or amplification by the polymerase chain reaction. The elements also might have been produced synthetically.

The term "pistil-specific" as used herein means expression confined to cells of the pistil - the stigma, the transmitting tissue of the style and ovary - and exclusive of any other cells in the plant body.

The term "anther-specific" as used herein means confined to cells of the anther - the tapetal cells, developing microspores, and pollen - and exclusive of any other cells in the plant body.

The term "minimal promoter" as used herein means a region from any promoter that provides a TFIID binding site (a TATA box) and a transcription initiation site.

As used herein, the phrase "functional equivalents" in the context of isolated DNA elements means DNA fragments that function the same, quanlitatively, as the first mentioned isolated DNA elements. Thus, if the isolated DNA element directs pistil-specific expression of a gene, a DNA fragment would be a functional equivalent if the DNA fragment also directed pistil-specific expression of a gene operably linked to the fragment in the same manner as the isolated DNA element. Quantitative equivalence is not needed for a fragment to be a functional equivalent according to this invention. Thus DNA fragments that have nucleotide substitutions, deletions and/or additions can be functional equivalents of an isolated DNA element.

In order to determine whether a DNA fragment is a "functional equivalent" of an isolated DNA element, the following assay(s) can be conducted. The promoter fragment is linked to a reporter gene such as β-glucuronidase or a toxic gene such as the diphtheria toxin subunit A, and introduced into plants. Promoter activity is analyzed by assays for β-glucuronidase activity or by visual inspection for toxic gene expression. These assays are described in Thorsness et al., Dev. Biol., Vol. 143, (1991) pages 173-184.

Construction of Isolated DNA Element that Directs Pistil- or Anther-Specific Expression

The isolated DNA elements of the present invention that direct pistil- or anther-specific expression can be constructed by PCR or by making deletions of suitable promoters, or by synthetic methods. For example, promoter fragments can be generated by PCR utilizing the *SLG* promoter of the S_{13} *SLG* allele as a template. The endpoints of these fragments relative to the translation initiation codon are -339 to from -143 to -79 for pistil-specific expression and -415 to -291 or -117 to -8 for pollen-specific expression. Promoter fragments also can be made from promoters of other alleles of the *SLG*, such as S_2, S_8 and R1, alleles of *SRK*, alleles of *SLR1*, and any other related or unrelated genes with expression patterns similar to *SLG* and *SLR1*. Preferably the promoters from which the promoter fragments are obtained contain sequence motifs of the five consensus sequences denoted as SEQ ID NOS:1-5. The endpoints of the promoter fragments can be determined based on sequence comparisons.

As will be more apparent from Example III herein, pistil- and anther-specific DNA elements

also can be patterned after parts of the sequences disclosed in Figures 3(A) and 3(B) (SEQ ID NOS:6-10), and especially those parts including the sequences found in the Boxes. Consensus sequences have been determined for each Box and are as follows: Box I, GACNAATGATA (SEQ ID NO:1); Box II, GTTTGT (SEQ ID NO:2); Box III, TGANTTAATCG (SEQ ID NO:3); Box IV, TGAAAAAGTCATNGA (SEQ ID NO:4); and Box V, ATTTTNCTTGTCTGCT (SEQ ID NO:5). In the consensus sequences N is A, T, C, G or a deletion.

Thus according to one embodiment of the present invention, the isolated DNA element that directs pistil-specific expression of a gene comprises three sequence motifs that have about 70% or more homology, i.e., sequence identity, to the three consensus sequences denoted as SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3, with greater degrees of homology, e.g., 80%, 90%, 95% or more being more preferred.

According to another embodiment of the present invention, the isolated DNA element that directs anther-specific expression of a gene comprises a sequence motif that has about 60% or more homology to the consensus sequence denoted as SEQ ID NO:5. Again, greater degrees of homology, e.g., 70%, 80%, 90%, 95% or more are preferred.

In an even further embodiment of the present invention, the isolated DNA element that directs anther-specific expression of a gene has about 60% or more, preferably 70%, 80%, 90%, 95% or more, homology to the -415 to -291 region of the sequence set forth in Figure 3(B) (SEQ ID NO:10).

Construction of Isolated DNA Element-Promoter Polypeptide or RNA-Encoding Gene Fusions

Methods are known in the art for construction of DNA elements operably linked to promoters.

(Sambrook et al, "Molecular Cloning, a Laboratory Manual", 2nd ed., Cold Spring Harbor Laboratory Press, N.Y., 1989 Vol. 1-3) Such methods could utilize an intermediate vector which can be any vector suitable for cloning and can be prepared by fusing a promoter, preferably a minimal promoter, to a polypeptide or RNA-encoding gene followed at the 3' end by a polyadenylation and transcription termination region of any plant gene.

Based on the orientation of the isolated DNA element relative to the restriction endonuclease sites of the vector, appropriate strategies can be taken to couple the isolated DNA element to the promoter and polypeptide or RNA-encoding gene.

The polypeptide or RNA-encoding gene can be any DNA sequence encoding a desired polypeptide or RNA. In a preferred embodiment of this invention, a gene that encodes a moiety cytotoxic to plant cells, or alternatively that perturbs pistil function or development, is linked to the female specific DNA element thereby causing female sterility. Similarly a gene that encodes a moiety cytotoxic to plant cells, or that perturbs anther function or development, is linked to the male specific DNA element thereby causing male sterility.

Examples of such genes are listed in European Patent Application Number 90402196.1, publication number 0 412 006 A1, filed July 31, 1990, the disclosure of which is hereby incorporated by reference.

Preferred examples of genes encoding moieties cytotoxic to plant cells include the genes which are described in the following references, the disclosures of which are hereby incorporated by reference:

a) Pectate lyase gene pelE from Erwinia chrysanthemi EC16, which degrades pectin, causing cell lysis. Kenn et al., J. Bacteriology, Vol. 168, (1986) page 595.

b) Diphtheria toxin A-chain gene (DTA), which inhibits protein synthesis, Greenfield et al., PNAS, U.S.A., Vol. 80, (1983) page 6853; Palmiter et al., Cell, Vol. 50, (1987) page 435.

c) T-urf13 (TURF-13) gene from cms-T maize mitochondrial genomes; this gene encodes a polypeptide designated URF13 which disrupts mitochondrial or plasma membranes. Braun et al., Plant Cell, Vol. 2, (1990) page 153; Dewey et al., PNAS, U.S.A., Vol. 84, (1987) page 5374; and Dewey et al., Cell, Vol. 44, (1986) page 439.

d) Gin recombinase gene from phage Mu gene, which encodes a site-specific DNA recombinase which will cause genome rearrangements and loss of cell viability when expressed in cells of plants. Maeser et al., Mol. Gen. Genet., Vol. 230, (1991) pages 170-176. A mutant gene, which is not host dependent, is also disclosed in Maeser et al.

e) Indole acetic acid-lysine synthetase gene (iaaL) from Pseudomonas syringae, which encodes an enzyme that conjugates lysine to indoleacetic acid (IAA). When expressed in the cells of plants, it causes altered development due to the removal of IAA from the cell via conjugation. Romano et al., Genes and Development, Vol. 5, (1991) pages 438-446; Spena et al., Mol. Gen. Genet., Vol. 227, (1991) pages 205-212; Robert et al., PNAS, U.S.A., Vol. 87, pages 5795-5801.

f) CytA toxin gene from Bacillus thuringiensis Israeliensis which encodes a protein that is mosquitocidal and hemolytic. When expressed in plant cells, it causes death of the cell due to disruption of the cell membrane.

McLean et al., J. Bacteriology, Vol. 169, (1987) pages 1017-1023; Ellar et al., United States Patent No. 4,918,006 (1990).

Especially preferred are constructions employing the peIE gene driven by an SLG_{13} promoter fragment that directs pistil-specific expression.

Other genes useful in creating female and male sterility include those encoding Adenine Phosphoribosyltransferase (APRT) (Moffatt and Somerville, Plant Physiol., Vol. 86, (1988) pages 1150-1154); DNase, RNase; proteases and salicylate hydroxylase.

It is further recognized that RNA which is capable of disrupting the formation of viable pollen or the flower, seed or embryo can be utilized in the present invention. The RNA of the invention includes anti-sense RNA as well as ribozymes.

Anti-sense RNA can be utilized which will hybridize with mRNA from a gene which is critical to pollen formation or function, e.g., APRT, or which is critical to flower, seed or embryo formation or function. In this manner, the anti-sense RNA will prevent expression of the necessary genes resulting in no pollen formation or no flower or seed formation, respectively.

Alternately, ribozymes can be utilized which target mRNA from a gene which is critical to pollen, flower, seed, or embryo formation or function. Such ribozymes will comprise a hybridizing region of at least about nine nucleotides which is complementary in nucleotide sequence to at least part of the target RNA and a catalytic region which is adapted to cleave the target RNA. Ribozymes are described in EPA No. 0 412 006, EPA No. 0 321 201 and WO88/04300 herein incorporated by reference. See, also Haseloff and

Gerlach, Nature, Vol. 334, (1988) pages 585-591; Fedor and Uhlenbeck, Proc. Natl. Acad. Sci., U.S.A., Vol. 87, (1990) pages 1668-1672; Cech and Bass, Ann. Rev. Biochem., Vol. 55, (1986) pages 599-629; T.R. Cech, Vol. 236, (1987) pages 1532-1539; T.R. Cech, Gene, Vol. 73, (1988) pages 259-271; and, Zang and Cech, Science, Vol. 231, (1986) pages 470-475.)

The construct may also include any other necessary regulators such as terminators, (Guerineau et al., Mol. Gen. Genet., Vol. 226, (1991) pages 141-144; Proudfoot, Cell, Vol. 64, (1991) pages 671-674; Sanfacon et al., Genes Dev., Vol. 5, (1991) pages 141-149; Mogen et al., Plant Cell, Vol. 2, (1990) pages 1261-1272; Munroe et al., Gene, Vol. 91, (1990) pages 151-158; Ballas et al., Nucleic Acids Res., Vol. 17, (1989) pages 7891-7903; Joshi et al., Nucleic Acid Res., Vol. 15, (1987) pages 9627-9639); plant translational consensus sequences (C.P. Joshi, Nucleic Acids Research, Vol. 15, (1987) pages 6643-6653), introns (Luehrsen and Walbot, Mol. Gen. Genet., Vol. 225, (1991) pages 81-93) and the like, operably linked to the nucleotide sequence. It may be beneficial to include 5′ leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation. Translational leaders are known in the art and include:

Picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5′ noncoding region) (O. Elroy-Stein, T.R. Fuerst and B. Moss, PNAS, U.S.A., Vol. 86, (1989) pages 6126-6130);

Potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Allison et al., (1986); MDMV leader (Maize Dwarf Mosaic Virus); Virology, Vol. 154, pages 9-20), and

Human immunoglobulin heavy-chain binding protein (BiP), (D.G. Macejak and P. Sarnow, Nature, Vol. 353, (1991) pages 90-94;

Untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4), (S.A. Jobling and L. Gehrke, Nature, Vol. 325, (1987) pages 622-625;

Tobacco mosaic virus leader (TMV), (D.R. Gallie et al., Molecular Biology of RNA, (1989) pages 237-256; and

Maize Chlorotic Mottle Virus leader (MCMV) (S.A. Lommel et al., Virology, Vol. 81, (1991) pages 382-385. See also, Della-Cioppa et al., Plant Physiology, Vol. 84, (1987) pages 965-968.

Signal sequences may be included within the expression cassette of the invention. Such signal sequences may be any DNA sequence which is able to direct the transport of an associated polypeptide. The signal sequence is preferably a sequence which is translated into a signal peptide, which becomes separated from the peptide after transit of the peptide is complete. Signal sequences are useful for directing the polypeptide product of the coding DNA sequence to a desired location within the cell, such as to the mitochondria or to the endoplasmic reticulum, or to direct extracellular transport outside of the cell. Among the signal sequences useful for the present invention are, for example, the signal sequence from the pathogenesis-related gene (PR-1) of tobacco, which is described in Cornellisen et al., EMBO, Vol. 5, (1986) pages 37-40; the yeast mitochondrial presequence; Schmitz et al., Plant Cell, Vol. 1, (1989) pages 783-791; the signal sequence from plant mitochondrial Rieske iron-sulfur protein, Huang et al., PNAS, U.S.A., Vol. 88, (1991) pages 10716-10720; mitochondrial and chloroplast (1989). The identification of

other leader sequences is known in the art. See Delle-Cioppa et al., Plant Physiology, Vol. 84, (1987) pages 965-968; Schekman, TIBS, (1985) page 188.

A plant terminator may be utilized in the expression cassette. See, Rosenberg et al., Gene, Vol. 56, (1987) page 125; Guerineau et al., Mol. Gen. Genet., Vol. 226, (1991) pages 141-144; Proudfoot, Cell, Vol. 64, (1991) pages 671-674; Sanfacon et al., Genes Dev., Vol. 5, (1991) pages 141-149; Mogen et al., Plant Cell, Vol. 2, (1990) pages 1261-1272; Munroe et al., Gene, Vol. 91, (1990) pages 151-158; Ballas et al., Nucleic Acids Res., Vol. 17, (1989) pages 7891-7903; Joshi et al., Nucleic Acid Res., Vol. 15, (1987) pages 9627-9639.

Plant Transformation

The aforementioned isolated DNA element-promoter-polypeptide or RNA-encoding-gene fusions can be introduced into an appropriate vector for transforming plants, e.g., agrobacteria, and the resulting bacterial strains can be used to transform plants to achieve a variety of effects, including female or male sterility.

Methodologies for the construction of plant expression cassettes as well as the introduction of foreign DNA into plants is generally described in the art. Generally, for the introduction of foreign DNA into plants Ti plasmid vectors have been utilized for the delivery of foreign DNA as well as direct DNA uptake, liposomes, electroporation, micro-injection, and the use of microprojectiles. Such methods have been published. See, for example, Guerche et al., Plant Science, Vol. 52, (1987) pages 111-116; Neuhause et al., Theor. Appl. Genet., Vol. 75, (1987) pages 30-

36; Klein et al., Nature, Vol. 327, (1987) pages 70-73; Howell et al., Science, Vol. 208, (1980) page 1265; Horsch et al., Science, Vol. 227, (1985) pages 1229-1231; DeBlock et al., Plant Physiology, Vol. 91, (1989) pages 694-701; Methods for Plant Molecular Biology, (Weissbach and Weissbach, eds.) Academic Press, Inc. (1988); and Methods in Plant Molecular Biology, (Schuler and Zielinski, eds.) Academic Press, Inc. (1989). It is understood that the method of transformation will depend upon the plant cell to be transformed.

It is further recognized that the components of the expression cassette may be modified to increase expression. For example, truncated sequences, nucleotide substitutions or other modifications may be employed.

As mentioned above, a preferred embodiment of the present invention is to use the isolated DNA elements to direct the expression of a gene that will kill or otherwise interfere with the function of pistil cells or anther cells. The pistil is the female reproductive structure of the plant, the function of which is to support germination and growth of the pollen tube until it reaches the ovule and can fertilize the egg cell. Interference with this function thus renders the plant female sterile. The ability to control the female fertility of plants is useful in the production of hybrid seed. The production of female sterile plants is described in European Patent Application number 90402196.1, Publication Number 0 412 006 A1 filed July 31, 1990, the disclosure of which is hereby incorporated by reference.

Briefly, the method of EP 0 412 006 A1 involves transforming a plant with a foreign DNA sequence which encodes a product which selectively disrupts the metabolism, functioning and/or

development of cells of the flowers. Pages 5-6 of the application list female-sterility DNAs any of which would be useful in the present invention.

Male sterile plants according to the present invention can be made by known methods analogous to those described for making female sterile plants. Male sterile plants are useful in breeding schemes for producing commercially desirable hybrids.

Plantlets resulting from independent transformation events can be propagated and analyzed. The presence of inserted DNA sequences in the putative transgenic plants can be verified by DNA blot analysis or PCR as described in the examples herein.

EXAMPLES

The invention will now be described by reference to specific examples, which are not meant to be limiting.

In the following examples base pair, i.e. nucleotide position, numbering of the SLG_{13} promoter follows that set forth in Figures 3(A) (SEQ ID NOS:6-9) and 3(B) (SEQ ID NO:10).

EXAMPLE I

CONSTRUCTION OF PROMOTER FRAGMENTS

Construction of 5′ Promoter Deletions

The starting point for the construction of sequential 5′ deletions of the SLG_{13} promoter was an SLG_{13} promoter-*GUS-nos* gene fusion consisting of a 3.65 kb promoter fragment fused to the β-glucuronidase reporter gene and to the polyadenylation signal and transcription termination region of the nopaline synthase *(nos)* gene of *Agrobacterium tumefaciens* (M.K. Thorsness, M.K. Kandasamy, M.E. Nasrallah and J.B. Nasrallah, Dev. Biol., Vol. 143, (1991) pages 173-184). The promoter fragment in this gene fusion extends from a natural BamH1 site 3.65 kb 5′ of the SLG_{13} protein-coding region to a BamH1 site that was introduced by site-directed mutagenesis at nucleotide -8 (8 bp upstream of the ATG initiating codon) in the SLG_{13} gene (M.K. Thorsness, M.K. Kandasamy, M.E. Nasrallah and J.B. Nasrallah, Dev. Biol., Vol. 143, (1991) pages 173-184). This SLG_{13}-*GUS-nos* fusion was cloned into the Bluescript (Stratagene) plasmid to generate plasmid pJSS1. pJSS1 was used to generate a deletion derivative to a natural HpaI site found in the SLG_{13} promoter at nucleotide -411. The resulting plasmid, pMKT29, was in turn used to generate two additional deletion derivatives: one in which sequences were deleted to the ClaI site at -151, and another in which sequences were deleted to the DraI site at -79.

Additional 5′ promoter deletions were generated by the polymerase chain reaction (PCR) with pMKT29 as template. Deletions were directed to positions -187 and to -117 in the SLG_{13} promoter by using, as 5′ amplimers, synthetic

oligonucleotides complementary to sequences at these positions, and as 3′ amplimer an oligonucleotide complementary to a sequence located within the β-glucuronidase coding region. The BamH1 site (at position -8 from the ATG) in the PCR-amplified fragments was used to insert the promoter fragments upstream of *GUS-nos*. The correct orientation and sequence of the fusions was verified by DNA sequence analysis. The endpoints of these derivatives (numbered from the translation initiation codon) and their respective orientations are given in Figure 1. Each of these promoter constructs retains the putative TATA-binding site found in the *SLG* promoter. A polyadenylation site is included at the terminus of the *GUS* coding region. The gene fusions were cloned into the binary vector pBIN19 (M. Bevan, Nucleic Acids Res., Vol. 12, (1984) pages 8711-8721).

Construction of Promoter Fragment-Minimal Promoter-*GUS* Fusions

Individual promoter modules were synthesized from the 5′ upstream region of the SLG_{13} allele by PCR and joined to the -46 to +8 region of the CaMV 35S promoter. This 35S promoter fragment provides the TFIID binding (TATA box) and transcription initiation sites, and has been used as a minimal promoter in studies of plant gene activity (P.N. Benfey, L. Ren and N.H. Chua, EMBO J., Vol. 9, (1990) pages 1677-1684). The endpoints of these fragments relative to the translation initiation codon are shown in Figure 2. The PCR-amplified products were cloned in the linearized plasmid vector pCR1000 (Invitrogen) and sequenced. Based on the orientation of the promoter fragments relative to the restriction endonuclease sites of the vector, one of two strategies was taken to

couple the *SLG* promoter domain to the minimal promoter and β-glucuronidase.

Plasmids that contained inserts facing "left" (i.e., towards the SpeI site of pCR1000) were opened at the SpeI site and joined to the intermediate vector *pEMBL-(GUS-46)-BS* at a corresponding SpeI site. In the resulting plasmid, the *SLG* promoter fragment is linked to the CaMV 35S minimal promoter-GUS fusion. The region of interest was excised from the plasmids as an EcoR1 fragment and cloned into the corresponding site of pBIN19.

The intermediate vector *pEMBL-(GUS-46)-BS* contained the -46 to +8 region of the CaMV 35S promoter joined to the coding region of the β-glucuronidase gene followed by the polyadenylation and transcription termination region of the ribulose-bis-phosphate carboxylase small subunit (rbcS) gene. This plasmid was generated by cloning a BglII-EcoR1 fragment of *pEMBL-(GUS-46)*, containing the CaMV 35S minimal promoter-*GUS-rbcS* terminator fusion, into the BamHI-EcoR1 sites of the Bluescript vector.

Plasmids that contained PCR-amplified products facing "right" were opened at a SacI site and fused at this site with *pEMBL-(GUS-46)-BS*. The resulting plasmids were digested with NotI and relegated to place the promoter fragments adjacent to the CaMV *35S-GUS-rbcS* fusion. These constructs were opened at an EcoR1 site and fused at the corresponding site to pBIN19.

EXAMPLE II
PLANT TRANSFORMATION

Plant Transformation

The aforementioned promoter-*GUS* fusions were introduced into *Agrobacterium tumefaciens* strain pCIB542/A136 (as one example of a vector for transforming plants). The pCIB542/A136 strain is derived from pEHA101 (E.E. Hood, G.L. Helmer, R.T. Fraley and M.-D. Chilton, J. Bacteriol., Vol. 168, (1986) pages 1291-1301) and the resulting bacterial strains were used to transform axenic excised leaf tissue of *N. tabacum cv.* Petit Havana as described (R.B. Horsch, J. Fry, N. Hoffman, J. Neidermeyer, S.G. Rogers and R.T. Fraley, In "Plant Molecular Biology Manual", S.B. Gelvin and R.A. Schilperoort, eds., Kluwer Academic Publishers, Dordrecht (1988) pages A5:1-9). Kanamycin-resistant plantlets resulting from independent transformation events were propagated and analyzed. The presence of inserted DNA sequences in the putative transgenic plants were verified in representative numbers of plants for each of the constructs by DNA blot analysis.

Histochemical Analysis

GUS activity was detected using the chromogenic substrate 5-bromo-4-chloro-3-indoyl-β-D-glucuronide (x-gluc) as described (R.A. Jefferson, T.A. Kavanagh and M.W. Bevan, EMBO J., Vol. 6, (1987) pages 3901-3907). Pistil sections that included the stigma and style were dissected longitudinally and incubated at 37°C in 2 mM x-gluc, 0.1 M NaPO4 (pH 7.0), 0.5% (v/v) Triton X-100 for 16 to 18 hrs. The tissue was destained in increasing concentrations of ethanol to a final concentration of 95% (v/v) ethanol and observed

under a dissecting microscope. Due to oxidative discoloration of the tissue surface, some of the samples were further dissected. Pollen grains were taken from several anthers of an open flower by tapping the grains into the well of a microtiter plate. Pollen was incubated in the above assay buffer at 37°C for 4 to 8 hrs., rinsed in 50% (v/v) ethanol, and viewed under a dissecting microscope. Sections of leaf, steam, root, petal, sepal, anther and filament tissue were analyzed as per pistil sections. Representative pollen and pistil sections were photographed under a phase contrast and dissecting microscope, respectively.

The results of this analysis are summarized in Table 1 below.

Table 1. Histochemical Localization of *GUS* Activity Conferred by *SLG* 5' Promoter Deletions and *SLG*-promoter Fragment-35S-GUS Fusions.

Construct Endpoints	TATA Element	Pistil Expression	Pollen Expression
- 411	*SLG*	5/7	5/7
- 187	*SLG*	0/18	0/18
- 187R	*SLG*	0/10	0/10
- 151	*SLG*	0/20	0/20
- 117	*SLG*	0/18	13/18
- 79	*SLG*	0/5	0/5
- 339 to - 79	35S	20/24	0/24
- 339 to - 143	35S	10/21	0/21
- 339 to - 163	35S	0/17	0/17
- 206 to - 79	35S	0/17	0/17
- 179 to - 79	35S	0/7	0/7
- 150 to - 79	35S	0/20	0/20
- 179 to - 143	35S	0/22	0/22
- 206 to - 143	35S	0/16	0/16
- 415 to - 291	35S	0/10	9/10
(none)	35S	0/6	0/6
wild type	-	0/4	0/4

Endpoints of promoter fragments are given in the first column. The TATA element used in a particular construction is indicated in the second column: *SLG* = native *SLG TATA element; 35S = -46 to +8 CaMV 35S minimal promoter. The number of plants exhibiting GUS* activity following staining with x-gluc is given over the number of independently isolated transgenic plants analyzed. "R" = reverse orientation.

Pistil-Specific Expression

A photograph of a longitudinal section through the upper region of a tobacco pistil showed that at the apex of the pistil was a bilobed stigma consisting of a papillate epidermis, a subepidermal zone, and parenchymatous ground tissue. The secretory zone of the stigma converged into the style as a central region of transmitting tissue surrounded by the cortex and the epidermis. During pollination, pollen germinates at the stigma surface, and the pollen tubes extend into the transmitting tissue of the style as they make their way towards the ovary. Untransformed tobacco pistils do not contain detectable endogenous *GUS* activity, and did not stain with x-gluc.

Tobacco plants transformed with three of the SLG_{13} promoter constructs exhibited *GUS* activity in the stigma and style (Table 1). From the first set of sequential 5′ promoter deletions, only one of the fragments, that having an endpoint at -411, directed *GUS* expression in the pistil. Activity was apparent along the length of the transmitting tissue of the style, in the stigma, and in papillar cells. *GUS* activity was detected in these tissues in each of the flower bud stages examined, ranging from buds 1.2 cm in length to open flowers. No activity was detected in leaf, root, anther, petal, sepal, or stem. The pattern of expression observed with the -411 construct was similar to that seen when a 3.65 kb *SLG* promoter-GUS fusion is introduced into *Nicotiana* (M.K. Thorsness, M.K. Kandasamy, M.E. Nasrallah and J.B. Nasrallah, Dev. Biol., Vol. 143, (1991) pages 173-184).

These data suggested that the DNA elements required for pistil-specific expression are located downstream of the -411 endpoint. The pistil-specifying ability of fragments with endpoints

within this region was assayed in the second set of constructions. Of the fragments joined to the minimal promoter and *GUS*, two tested positive for *GUS* activity. A region spanning from -339 to -79 and a 196 bp fragment with endpoints at -339 and -143 gave results that were very similar to the -411 construct described above. Expression was detected in the stigma secretory zone, stylar transmitting tissue, and in stigma papillar cells. However, the intensity of expression from the shorter (-339 to -143) fragment was noticeably less than that from the -411 and the -339 to -79 constructs. In addition, fewer of these transformed plants stained positive for *GUS* activity: blue staining was noted in 5 of 7 plants transformed with the -411 fragment and in 20 of 24 plants transformed with the -339 to -79 fragment, but in only 10 of 21 plants transformed with the shorter fragment (Table 1). *GUS* activity was detected in pistils of open flowers and in buds as small as 1.6 cm in length and was not detected in other floral or vegetative tissues. Narrowing the 3′ end of the 196 bp fragment by only 20 bp to -163 or decreasing the 5′ end to -206 leads to the loss of *GUS* expression (Table 1). Plants transformed with a construct containing the -46 to +8 CaMV 35S minimal promoter region linked alone to *GUS* or with a 5′ deletion having the -187 fragment in reverse orientation (-187R) did not show *GUS* activity in pistils (Table 1).

Pollen-Specific Expression

Three of the constructs allow for consistent *GUS* expression in pollen (Table 1). Of the sequential promoter deletions, both the -411 and -117 constructs showed activity in pollen, while constructs with endpoints in between did not have

activity (Table 1). Of the 35S minimal promoter fusions, one construct, with endpoints of -415 and -291, exhibited *GUS* activity in pollen. Other than the -411 deletion, none of these constructs conferred expression in the pistil. Conversely, the 196 bp pistil-specific domain and the pistil-specifying -339 to -79 *35S-GUS* fusion did not exhibit consistent pollen expression (Table 1). On occasion, light-blue staining was noted for pollen of plants containing other constructs; however, this staining was not evident in further screenings of these plants.

For each of the pollen expressing constructs, expression was noted only in pollen of open flowers and was not seen in pollen from flowers of earlier stages of development. Staining was limited to pollen and was not detected in the sporophytic tissue of the anther. In some of the plants, approximately one half of the pollen grains showed *GUS* activity, consistent with the introduction of a single copy of the gene into the genome of these plants. For several of the plants, expression in pollen varied, and may, perhaps, be influenced by an unknown environmental factor. Staining was not observed in pollen of untransformed plants or in pollen of plants transformed with the 35S minimal promoter fused alone to *GUS* (Table 1).

EXAMPLE III

DETERMINATION OF THE NUCLEOTIDE SEQUENCE OF *SLG* AND *SLR1* PROMOTER REGIONS

The isolation of *SLG* alleles was previously described: SLG_{13} was isolated from a *B. oleracea cv. acephala* (kale) S_{13} homozygote (J.B. Nasrallah, S.M. Yu and M.E. Nasrallah, Proc. Natl. Acad. Sci., U.S.A., Vol. 85, (1988) pages 5551-5555); SLG_2 from

a *B. oleracea cv. alboglabra* S_2 homozygote (C.H. Chen and J.B. Nasrallah, Mol. Gen. Genet., Vol. 127, (1990) pages 221-228); SLG_8 from the *B. campestris* S_8 homozygote (K. Toriyama, J.C. Stein, M.E. Nasrallah and J.B. Nasrallah, Theor. Appl. Genet., Vol. 81, (1991) pages 769-776). The *SLR1* gene was isolated from a *B. oleracea cv. acephala* S_{22} homozygote as follows. Genomic DNA was prepared from leaf tissue, purified on CsCl gradients, digested with EcoR1, and fractionated on agarose gels. The region of the gel that contained the *SLR1* gene, previously identified by DNA gel blot analysis, was excised from the gel. The DNA was eluted and used to construct a sub-genomic library in the bacteriophase λGEM11 (Stratagene). *SLR1*-containing clones were identified by hybridization to an *SLR1*-cDNA probe (B.A. Lalonde, M.E. Nasrallah, K.G. Dwyer, C.H. Chen, B. Barlow and J.B. Nasrallah, Plant Cell, Vol. 1, (1989) pages 249-258).

For nucleotide sequence analysis, restriction fragments containing sequences 5′ of the initiating ATG codon were subcloned into appropriate plasmid vectors. For the SLG_{13} and SLG_2 genes, restriction fragments were subcloned into the M13 vectors mp18 and mp19 (C. Yanisch-Perron, J. Vieira and J. Messing, Gene, Vol. 33, (1985) pages 103-119) and single-stranded DNA templates were sequenced by the dideoxynucleotide chain termination method (F. Sanger, S. Nicklen and A.R. Coulson, Proc. Natl. Acad. Sci., U.S.A., Vol. 74, (1977) pages 5463-5467). For the SLG_8 and *SLR1* genes, restriction fragments were subcloned into the pUC118 and pUC119 vectors (J. Vieira and J. Messing, Methods Enzymol., Vol. 153, (1987) pages 3-11). A series of nested deletions were generated by ExoIII digestion using the Erase-A-

Base kit (Promega), and the DNA sequence was determined on double-stranded plasmid templates (E.Y. Chen and P.H. Seeburg, DNA, Vol. 4, (1985) pages 165-170).

The nucleotide sequences of the promoter regions of 3 alleles of the *Brassica SLG* gene -- SLG_{13} and SLG_2 from *B. oleracea*, and SLG_8 of *B. campestris* -- and one allele of the *SLR1* gene were determined. The S-locus related gene, *SLR1*, is a member of the S-gene family that is unlinked to the S-locus, and is expressed in a manner similar to *SLG* (B.A. Lalonde, M.E. Nasrallah, K.G. Dwyer, C.H. Chen, B. Barlow and J.B. Nasrallah, Plant Cell, Vol. 1, (1989) pages 249-258; M. Trick and R.B. Flavell, Mol. Gen. Genet., Vol. 218, (1989) pages 112-117; R.M. Hackett, M.J. Lawrence and F.C.H. Franklin, Plant J., Vol. 2, (1992), pages 613-617). The four promoter regions share a high degree of sequence homology with five elements being particularly conserved. These five boxes are shown aligned in Figure 3(A) (SEQ ID NOS:6-9) and Boxes I to V have the consensus sequences denoted as: SEQ ID NO:1; SEQ ID NO:2; SEQ ID NO:3; SEQ ID NO:4; and SEQ ID NO:5, respectively. Within each box, actual sequence homology is about 70% for Box I, about 80% for Box II, about 80% for Box III, about 50% for Box IV and about 60% for Box V. In addition, several stretches of sequence similarity are found between individual genes. For example, the SLG_{13} and SLG_8 alleles are 85% identical over 202 bp, while the SLG_2 allele is only 66% identical to SLG_{13} over 289 bp. The SLR_1 sequence is the least conserved and shares approximately 40% sequence identity with the SLG_2 and SLG_{13} alleles.

Figure 4 is a diagram showing the arrangement of the functional elements within the SLG_{13} promoter.

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Thorsness, Mary K.

(ii) TITLE OF INVENTION: ISOLATED DNA ELEMENTS THAT DIRECT
PISTIL-SPECIFIC AND ANTHER-SPECIFIC GENE EXPRESSION
AND METHODS OF USING SAME

(iii) NUMBER OF SEQUENCES: 10

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

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(C) TELEX: 6491103

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GACNAATGATA

11

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GTTTGT

6

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGANTTAATCG

11

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGAAAAAGTC ATNGA

15

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATTTTNCTTG TCTGCT

16

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 289 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATTTTCTAAC AGACTTAGAT GCACTTGCGA ACAACATACT
TGCTGAACAC CATATGTTAT 60

GTTGGCAGGG TGAGAAATTA ATCACGTGTA GATATAGAAG
TAGTAGACAA ATGATATAGG 120

TTTGTGGGAA TGAATTAATC GATGGGATGA AAAAGTCATC
GAACATGTAA CACCACATTT 180

TACTTGTCTG CTAGGTTCGT GATAGTCGTT TAAATTAGAT
ACGTGAAAAA AGATTATAAA 240

TATGCAAAAG GGGAAGGGGA AGAAAAGAAA GAAAAAGGAG
GGGAGAGAA 289

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 311 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ACTTGAATGT ATCGAATCAT ACTATTGAGA CCACCATACT
TGCGGAATAC CATATGCTAT 60

GTTGACAGCG TGAGAACTAA TAACGTGTAG ATATAAAAGT
AGTTGACTGA ATGATACAAG 120

TTTGTGGAAG TGACTTAATC GTAGGGATGA AAAAGTCATG
GACTATGGAA CACAACATTT 180

TGCTTGCCAG TTAGGTTCGT CATAATAGTT TAATTCGAAT
TTTCTTGCAA AGTAACTTAG 240

GATGTATATA TATGTGCAAG TAGGACAAAA ACTAACAACA
AGAAAAAAAA AGAAAGAAAG 300

TGGTGGGGAA A
311

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 202 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTAGTAGGTA TAAAAGTAGT GGACAAATGA TACACGTTTT
TGGAAATGAA TTAATCGATG 60

GGATGAAAAA GTCATCGAAC ATGTAACACC ACATTTTGCT
TGTCTGCTAG GTTCCTTATA 120

GTCGTTTAAA ATCTGTATGT GGAAAAGATT ATAAATAAGC
AAGGGGAGGG GGAAAGAAAG 180

AAAGAACAAG GTGGGGAGAG AA
202

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 356 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TTTTAAGTCA AACTGAAGGA AACAACATAT GATATGTTAT
GTCATTTGGT CCAAAAACAC 60

AATGTTACGT TGCATGAGAA ATCAATTTCA CGTGGTAAGG
TTACTGACCA ATGACAATAG 120

TTTGTTAAAA TGAGTTAATG AGTGGCTGGA AAGTCATAGA
ATGTGGAAAT AAAAAATTTT 180

CTTGTCTGCT GGAAAGTATA TAATATCTAC AATTAAGACA
TAAACCATGC AAATTAAAAT 240

CAAACCATCC TCATTAGGTT TGCAAATCTA ATAAAGACAT
AAAGTCCATA TGTAACAATT 300

TTTTTCTATA AATAACGGGC GACAATGCAT AGAAAATTAA
AGTGGTGAAG AGAGAG 356

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 186 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TGCTGAGATG GATTTACAAT TTGATTTCTT TTGTATTTTT
ATTTGGTGTG TTTAATATAT 60

TAGTTAACCA ATTTACGTTA TACCAAATTT TTCAACCCTC
TTTTTAGTAA AAAACGAAAT 120

TAAAGTTTTT TCCCTCTTAG TCCGACGATT TTAAGCTAAT
TAGTTCGAAC AAAGAGTACA 180

ACATTA
186

What Is Claimed Is:

1. An isolated DNA element that directs pistil-specific expression of a nucleotide sequence that is operably linked to a minimal promoter that is operably linked to said isolated DNA element; functional equivalents of said isolated DNA element; and DNA fragments having sequences complementary to said isolated DNA element or its functional equivalents.

2. The isolated DNA element of claim 1, wherein said element comprises three sequences that have about 70% or more homology to three consensus sequences, respectively, denoted as SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3; functional equivalents of said isolated DNA element; and DNA fragments having sequences complementary to said isolated DNA element or its functional equivalents.

3. The isolated DNA element of claim 2, wherein said homology is about 80% or more.

4. The isolated DNA element of claim 2, wherein said homology is about 80% or more; functional equivalents of said isolated DNA element; and DNA fragments having sequences complementary to said isolated DNA element or its functional equivalents.

5. The isolated DNA element of claim 4, wherein said homology is about 90% or more; functional equivalents of said isolated DNA element; and DNA fragments having sequences complementary to said isolated DNA element or its functional equivalents.

6. The isolated DNA element of claim 2, wherein the entire sequence of said element that comprises said three sequences corresponds to a part of one of the sequences selected from the group consisting of SEQ ID NO:6; SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; functional equivalents of said isolated DNA element; and DNA fragments having sequences complementary to said isolated DNA element or its functional equivalents.

7. The isolated DNA element of claim 3, wherein the entire sequence of said element that comprises said three sequences corresponds to a part of one of the sequences selected from the group consisting of SEQ ID NO:6; SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; functional equivalents of said isolated DNA element; and DNA fragments having sequences complementary to said isolated DNA element or its functional equivalents.

8. The isolated DNA element of claim 4, wherein the entire sequence of said element that comprises said three sequences corresponds to a part of one of the sequences selected from the group consisting of SEQ ID NO:6; SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; functional equivalents of said isolated DNA element; and DNA fragments having sequences complementary to said isolated DNA element or its functional equivalents.

9. The isolated DNA element of claim 5, wherein the entire sequence of said element that comprises said three sequences corresponds to a part of one of the sequences selected from the group consisting of SEQ ID NO:6; SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; functional equivalents of said isolated DNA element; and DNA fragments having

sequences complementary to said isolated DNA element or its functional equivalents.

10. The isolated DNA element of claim 2, wherein the part of one of the sequences is from SEQ ID NO:6; functional equivalents of said isolated DNA element; and DNA fragments having sequences complementary to said isolated DNA element or its functional equivalents.

11. The isolated DNA element of claim 6, wherein the part of one of the sequences is from SEQ ID NO:6; functional equivalents of said isolated DNA element; and DNA fragments having sequences complementary to said isolated DNA element or its functional equivalents.

12. The isolated DNA element of claim 10, which is the sequence extending from base pair -339 to any of base pairs -143 to -79 of SEQ ID NO:6 and SEQ ID NO: 10; functional equivalents of said isolated DNA element; and DNA fragments having sequences complementary to said isolated DNA element or its functional equivalents.

13. The isolated DNA element of claim 12, wherein the sequence extends to base pair -143; functional equivalents of said isolated DNA element; and DNA fragments having sequences complementary to said isolated DNA element or its functional equivalents.

14. The isolated DNA element of claim 12, wherein the sequence extends to base pair -79; functional equivalents of said isolated DNA element; and DNA fragments having sequences

complementary to said isolated DNA element or its functional equivalents.

15. A plant having integrated into its genome the isolated DNA element of any one of claims 1, 2, 3, 4, or 9, or functional equivalents of the isolated DNA element, operably linked to a gene that encodes a polypeptide or RNA.

16. The plant of claim 15, wherein the at least one gene encodes a polypeptide or RNA that causes female sterility in plants.

17. The plant of claim 16, wherein said at least one gene that encodes a polypeptide that causes female sterility in plants, encodes one member selected from the group consisting of pectate lyase gene pelE from Erwinia chrysanthemi EC16, Diphtheria toxin A-chain gene, T-urf 13 gene from cms-T maize mitochondrial genomes, gin recombinase gene from phage Mu gene, indole acetic acid-lysine synthetase gene from Pseudomonus syringae, and CytA toxin gene from Bacillus thuringiensis Israeliensis.

18. The plant of claim 17, wherein said at least one gene encodes the pectate lyase gene PelE from Erwinia chrysanthemi EC16.

19. The plant of claim 16, wherein said at least one gene that encodes a polypeptide that causes female sterility in plants encodes one member selected from a group consisting of adenine phosphoribosyltransferase, DNase, RNase, protease and salicylate hydroxylase.

20. The plant of claim 16, wherein said at least one gene that encodes a polypeptide that causes female sterility in plants encodes a ribozyme.

21. The plant of claim 16, wherein said gene that encodes RNA that causes female sterility in plants encodes anti-sense RNA.

22. A method of selectively expressing a gene in a plant pistil, said method comprising growing a plant having integrated into its genome the isolated DNA element of any one of claims 1, 2, 3, 4, or 9, or functional equivalents of the isolated DNA element, operably linked to said gene.

23. A method of producing a female sterile plant, said method comprising growing a plant having integrated into its genome the isolated DNA element of any one of claims 1, 2, 3, 4, or 9, or functional equivalents of the isolated DNA element, operably linked to a gene that encodes a polypeptide or RNA that causes female sterility in plants.

24. The method of claim 23, wherein said at least one gene that encodes a polypeptide that causes female sterility in plants, encodes one member selected from the group consisting of pectate lyase gene pelE from Erwinia chrysanthemi EC16, Diphtheria toxin A-chain gene, T-urf 13 gene from cms-T maize mitochondrial genomes, gin recombinase gene from phage Mu gene, indole acetic acid-lysine synthetase gene from Pseudomonus syringae, and CytA toxin gene from Bacillus thuringiensis Israeliensis.

25. The method of claim 24, wherein said at least one gene encodes the pectate lyase gene PelE from Erwinia chrysanthemi EC16.

26. The method of claim 23, wherein said at least one gene that encodes a polypeptide that causes female sterility in plants encodes one member selected from a group consisting of adenine phosphoribosyltransferase, DNase, RNase, protease and salicylate hydroxylase.

27. The method of claim 23, wherein said at least one gene that encodes polypeptide that causes female sterility in plants encodes a ribozyme.

28. The method of claim 23, wherein said gene that encodes RNA that causes female sterility in plants encodes anti-sense RNA.

29. An isolated DNA element that directs anther-specific expression of a nucleotide sequence that is operably linked to a minimal promoter that is operably linked to said isolated DNA element, wherein said element comprises a sequence that has about 60% or more homology to a consensus sequence denoted as SEQ ID NO:5; functional equivalents of said isolated DNA element; and DNA fragments having sequences complementary to said isolated DNA element or its functional equivalents.

30. The isolated DNA element of claim 29, wherein said element comprises a sequence that has about 60% or more homology to a consensus sequence denoted as SEQ ID NO:5; functional equivalents of said isolated DNA element; and DNA fragments having sequences complementary to said isolated DNA element or its functional equivalents.

31. The isolated DNA element of claim 29, wherein said homology is about 70% or more.

32. The isolated DNA element of claim 31, wherein said homology is about 90% or more.

33. The isolated DNA element of claim 29, wherein the entire sequence of said element corresponds to a part of one of the sequences selected from a group consisting of SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; functional equivalents of said isolated DNA element; and DNA fragments having sequences complementary to said isolated DNA element or its functional equivalents.

34. The isolated DNA element of claim 30, wherein the entire sequence of said element corresponds to a part of one of the sequences selected from a group consisting of SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9; functional equivalents of said isolated DNA element; and DNA fragments having sequences complementary to said isolated DNA element or its functional equivalents.

35. The isolated DNA element of claim 33, wherein the part of one of the sequences is from SEQ ID NO:6; functional equivalents of said

isolated DNA element; and DNA fragments having sequences complementary to said isolated DNA element or its functional equivalents.

36. The isolated DNA element of claim 35, which has the sequence extending from base pair -117 to -8; functional equivalents of said isolated DNA element; and DNA fragments having sequences complementary to said isolated DNA element or its functional equivalents.

37. The isolated DNA element of claim 29, wherein said element comprises a sequence that has about 60% or more homology to a sequence extending from base pair -415 to -291 of the sequence denoted as SEQ ID NO:10; functional equivalents of said isolated DNA element; and DNA fragments having sequences complementary to said isolated DNA element or its functional equivalents.

38. The isolated DNA element of claim 29, wherein said element comprises a sequence that has about 60% or more homology to a sequence extending from base pair -415 to -291 of the sequence denoted as SEQ ID NO:10; functional equivalents of said isolated DNA element; and DNA fragments having sequences complementary to said isolated DNA element or its functional equivalents.

39. The isolated DNA element of claim 37, wherein said homology is about 70% or more.

40. The isolated DNA element of claim 39, wherein said homology is about 90% or more.

41. The isolated DNA element of claim 40, wherein the entire sequence of said element is the

sequence extending from base pair -415 to -291 of SEQ ID NO:10; functional equivalents of said isolated DNA element; and DNA fragments having sequences complementary to said isolated DNA element or its functional equivalents.

42. A plant having integrated into its genome the isolated DNA element of any of claims 29, 30, 33, 35, 37, 38 or 41, or functional equivalents of the isolated DNA element, operably linked to a gene.

43. The plant of claim 42, wherein the at least one gene encodes a polypeptide or RNA that causes male sterility in plants.

44. The plant of claim 43, wherein said at least one gene that encodes a polypeptide that causes male sterility in plants, encodes one member selected from the group consisting of pectate lyase gene pelE from Erwinia chrysanthemi EC16, Diphtheria toxin A-chain gene, T-urf 13 gene from cms-T maize mitochondrial genomes, gin recombinase gene from phage Mu gene, indole acetic acid-lysine synthetase gene from Pseudomonus syringae, and CytA toxin gene from Bacillus thuringiensis Israeliensis.

45. The plant of claim 44, wherein said at least one gene encodes the pectate lyase gene PelE from Erwinia chrysanthemi EC16.

46. The plant of claim 43, wherein said at least one gene that encodes a polypeptide that causes male sterility in plants encodes one member selected from a group consisting of adenine

phosphoribosyltransferase, DNase, RNase, protease and salicylate hydroxylase.

47. The plant of claim 43, wherein said at least one gene that encodes polypeptide that causes male sterility in plants encodes a ribozyme.

48. The plant of claim 43, wherein said gene that encodes RNA that causes male sterility in plants encodes anti-sense RNA.

49. A method of selectively expressing a gene in plant anthers, said method comprising growing a plant having integrated into its genome the isolated DNA element of any one of claims 29, 30, 33, 35, 37, 38 or 41, or functional equivalents of the isolated DNA element, operably linked to a gene.

50. A method of producing a male sterile plant, said method comprising growing a plant having integrated into its genome the isolated DNA element of any one of claims 29, 30, 33, 35, 37, 38 or 41, or functional equivalents of the isolated DNA element, operably linked to a gene that encodes a polypeptide or RNA that causes male sterility in plants.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/04557

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C12N 15/82; C07H 21/04; A01H 5/00
US CL : 435/172.3; 536/24.1; 800/205
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3; 536/24.1; 800/205

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, GenBank, UMBL
search terms: pollen, stigma, style, anther, pistil, promoter

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	Developmental Biology, Volume 143, issued 1991, Thorsness et al, "A *Brassica* S-Locus gene promoter targets toxic gene expression and cell death to the pistil and pollen of transgenic *Nicotiana*", pages 173-184, see entire document.	1-17, 22-24, 29-44, 49-50 ---------- 18-21, 45-48
X,P ----- Y,P	The Plant Cell, Volume 5, issued August 1993, Dzelzkalns et al, "Distinct *cis*-acting elements direct pistil-specific and pollen-specific activity of the Brassica S Locus glycoprotein gene promoter", pages 855-863, see entire document.	1-15, 29-42 ---------- 16-28, 43-50

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 JULY 1994	JUL 28 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer P. Moody Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/04557

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	The Plant Journal, Volume 2, Number 4, issued 1992, Hackett et al, "A *Brassica* S-Locus related gene promoter directs expression in both pollen and pistil of tobacco", pages 613-617, see entire document.	1-15, 29-42 ---------- 16-28, 43-50
X ---- Y	Developmental Biology, Volume 143, issued 1991, Toriyama et al, "A *Brassica* S locus gene promoter directs sporophytic expression in the anther tapetum of transgenic *Arabidopsis*", pages 427-431, see entire document.	1-15, 29-42 ---------- 16-28, 43-50
X ---- Y	The Plant Cell, Volume 3, issued September 1991, Sato et al, "Activity of an S locus gene promoter in pistils and anthers of transgenic Brassica", pages 867-876, see entire document.	1-15, 29-42 ---------- 16-28, 43-50
X ---- Y	EP, A, 0,436,467 (NASRALLAH ET AL) 10 July 1991, see entire document.	1-17, 22-24, 29-44, 49-50 ---------- 18-21, 25-28, 45-48
Y	EP, A, 0,412,006 (DE GREEF ET AL) 06 February 1991, see entire document.	16-28

INTERNATIONAL SEARCH REPORT

[redacted]ational application No.
PCT/US94/04557

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Telephone Practice
Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims. it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-28, drawn to a pistil-specific first DNA, plants containing same and method of use; Classes 435, 536, and 800; Subclasses 172.3, 24.1, and 205, respectively, for example.
II. Claims 29-50, drawn to an anther-specific second DNA, plants containing same and method of use; Classes 435, 536, and 800; Subclasses 172.3, 24.1, and 205, respectively, for example.

The DNA regulatory element, plants and methods of use of Groups I and II are each capable of separate manufacture and use and have different properties as claimed. The products perform completely different operations and are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single general inventive concept. Because these inventions are distinct for the reasons given above and since they have acquired a separate status in the art as shown by their different subject matter and are separately searched, a holding of lack of unity as indicated is proper.